LUNG BREATHING IN THE BULLFROG: GENERATING RESPIRATORY RHYTHM AND PATTERN

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A

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ABSTRACT

This research investigated location of the lung respiratory rhythm generator (RRG) in the bullfrog brainstem using neurokinin-1 (NK1R) and μ-opioid (μOR) receptor colocalization and characterized the role of these receptors in breathing pattern formation. Colocalization was distinct near the facial nucleus in juvenile bullfrogs but not in tadpoles. NK1R intensity exhibited no developmental change, while μOR intensity increased from late-stage tadpoles to juvenile frogs. Substance P (NK1R agonist; bath applied) increased lung burst frequency, lung burst cycle frequency (BCF), episode frequency, lung burst amplitude and area, but decreased number of lung bursts per episode and lung burst duration. Antagonist D decreased lung burst frequency and BCF, episode frequency, and the number of lung bursts per episode, and increased lung burst duration and area. DAMGO (μOR agonist; bath applied) decreased lung burst frequency and BCF, episode frequency, and number of lung bursts per episode, but increased all lung burst parameters. Naloxone (μOR antagonist) increased lung burst frequency and BCF, episode frequency, lung bursts per episode but decreased all lung burst parameters. Together these results indicate that NK1R and μOR colocalization represents the lung RRG, and that episode formation is intrinsic to the respiratory control network but may or may not originate in the RRG.

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GENERAL INTRODUCTION

Neural control of breathing has been most extensively investigated in mammals, which typically breathe in a pattern of continuous lung ventilation. Two distinct neuronal networks are proposed to be essential for respiratory rhythm generation in mammals (Onimaru *et al.*, 1997; Rekling & Feldman, 1998): inspiratory neurons in the preBötzinger complex (PBC; Smith *et al.*, 1991; Gray *et al.*, 2001) and pre-inspiratory neurons in the parafacial respiratory group (pFRG; Onimaru *et al.*, 1995). These two neuronal networks interact to produce a continuous rhythmic motor pattern. Neuronal activity in both these areas is influenced by a variety of chemical agents including, but not limited to: acetylcholine, GABA, glutamine, glutamate and aspartate (Metz, 1966; Folbergrova *et al.*, 1975; Weyne *et al.*, 1978) during hypercapnia; GABA, glutamate, glycine and taurine (Kazemi & Hoop, 1991) during hypoxia; as well as somatostatin (Chen *et al.*, 1990; Llona *et al.*, 2004), thyrotropin-releasing hormone (Rekling, 1990; Bayliss *et al.*, 1992) and adenosine (Dong & Feldman, 1995). Mammalian breathing, that outwardly simple pattern of continuous lung ventilation, is thus the result of a complex synchronicity of signaling within a neural network that encompasses the PBC and pFRG.

The mammalian PBC contains a high density of neurokinin-1 receptor- (NK1R) expressing neurons (Gray *et al.*, 1999). These neurons are necessary for maintaining a normal respiratory rhythm (Gray, 2001; Wang *et al.*, 2002) and are located in all chemosensory sites (Nattie & Prabhakar, 2001; Nattie & Li, 2002). The PBC also contains a large number of neurons expressing substance P (SP) and synapsing with 1/3 of NK1R-expressing neuron dendrites (Liu *et al.*, 2004). NK1R are the preferred binding site for SP (Makeham *et al.*, 2001), and local application of SP to the PBC increases respiratory frequency in rats (Gray *et al.*, 1999).

Opiates decrease respiratory frequency and inhibit the recruitment of PBC inspiratory neurons by pFRG neurons, but have no effect on pre-inspiratory pFRG neurons (Takeda

et al., 2001). Mammalian PBC inspiratory neurons express μ-opioid receptors (μOR; Gray *et al*, 1999). Colocalization of NK1R and μOR has been used to determine the location of the PBC in mammalian brainstem slices (Gray *et al.*, 1999).

In contrast to mammals, adult anurans exhibit two distinct rhythmic motor patterns: rhythmic buccal ventilation, a remnant of gill ventilation that does not contribute to gas exchange (Winmill & Hedrick, 2003); and lung ventilation, which often occurs in episodes (Kinkead & Milsom, 1994). The rhythmic, episodic, bimodal pattern of breathing of frogs is proposed to be the combined output of two respiratory rhythm generators (RRG), one for buccal and one for lung ventilation (Wilson *et al.*, 2002). The mechanisms generating lung ventilation in the frog are believed to be homologous to those generating breathing in mammals (Vasilakos *et al.*, 2005; Wilson *et al.*, 2006), despite the disparity of episodic breathing occurring in frogs. In intact adult frogs and isolated brainstems prepared from juveniles, opiates depress lung burst frequency while buccal rhythm remains constant (Vasilakos *et al.*, 2005); this is one line of evidence suggesting parity between the mammalian PBC and the frog lung RRG.

In amphibians, lung ventilation often occurs in episodes (Kinkead & Milsom, 1994). Episodic lung ventilation is characterized by ventilatory activity consisting of a series of lung breaths in a cluster, and separated from subsequent lung-breath clusters by non-ventilatory activity of apnea and/or continuous buccal ventilation. Episodes consist of variable numbers of individual breaths (Kinkead & Milsom, 1994, 1996, 1997). This pattern is complex; it can vary in the duration of episodes, the number of breaths per episode, and the duration of the non-ventilatory activity between episodes. Previous studies investigating the mechanisms underlying episodic breathing examined central and peripheral chemoreceptors (West *et al.*, 1987; Smatresk & Smits, 1991; Kinkead & Milsom, 1994), pulmonary stretch receptors (Kinkead & Milsom, 1996, 1997) and olfactory receptors (Kinkead & Milsom, 1996). It was shown that each receptor group

influenced breathing pattern by altering the number of breaths per episode, the duration of the non-ventilatory activity between episodes, or both. None of the receptor groups was shown to be directly responsible for episode formation (Kinkead, 1997), and it was concluded that the mechanisms responsible for pattern formation might be an intrinsic property of the central respiratory control system (Kinkead *et al.*, 1994). This idea supports the hypothesis that, in episodic breathing, the episode is the fundamental output of the control system rather than the individual breath within the episode (Jackson, 1978).

Identification of brain area responsible for episodic pattern formation has been attempted, but with limited success. Transecting the brainstem between the optic lobes and cerebellum eliminates episodes consisting of more than one breath in the bullfrog, and the breathing pattern appears to consist of evenly spaced breaths (Oka, 1958a, b). This indicates that episode formation is under the influence of a site independent of that responsible for respiratory rhythm formation. Studies using brainstem-spinal cord preparations of bullfrogs (Kinkead *et al.*, 1994), turtles (Douse & Mitchell, 1990) and neonatal rats (Hilaire *et al.*, 1989) have demonstrated the occurrence of episodic breathing *in vitro*. Identifying changes in pattern formation can be difficult, as changes may occur secondarily due to overall changes in ventilatory drive. Studies with baclofen (Straus *et al.*, 2000b) and nitric oxide (Harris *et al.*, 2002) have shown that pattern formation can be altered independently of overall rhythm generation. These studies measured cranial nerve output that would activate the respiratory-related muscles, and lacked sensory afferents and descending inputs, indicating that the production of episodes is an intrinsic property of the respiratory control network.

The aim of this study was to use double-label immunofluorescence techniques to identify colocalization of NK1R and μOR. Such colocalization may indicate a location of the putative lung RRG in the juvenile bullfrog, a location homologous to the mammalian PBC. Immunofluorescence experiments performed on tadpoles of different

developmental stages investigated the ontogeny of the putative lung RRG. The influences of SP, antagonist D, [D-Ala^2, N-Me-Phe^4, Gly^5-ol] (DAMGO) and naloxone on respiratory rhythm were also characterized. This characterization justified interpreting the colocalization of NK1R and μOR as a marker of the putative lung RRG and investigated the role of these receptors in respiratory pattern generation. Determining the neural locations responsible for episodic pattern formation has proven elusive. This study, by investigating the role of NK1R and μOR in the formation of episodic pattern, provides new insights.

Chapter 1:

μ-opioid and neurokinin-1 receptor immunofluorescence and involvement in the neuroventilation of bullfrogs[1]

Abstract

Investigation of functional homologies between anuran and mammalian respiratory rhythm generators (RRGs) has been limited by difficulty in defining the sites and neuronal composition of the RRGs. μ-opioid receptor (μOR) and neurokinin-1 receptor (NK1R) colocalization has been used as a method of identifying the preBötzinger complex, an area proposed as a RRG in mammals. I conducted immmunocytochemical staining for μOR and NK1R of *Lithobates* (formerly *Rana*) *catesebeiana* brainstem slices to determine if a similar region or regions could be identified in the bullfrog. I identified one distinct site of μOR and NK1R colocalization in the region of the bullfrog facial nucleus, an area purportedly involved in anuran respiratory rhythm generation. Bath application of the μOR agonist DAMGO (100 nM) to the isolated brainstem preparation significantly depressed lung burst frequency ($P < 0.001$). Bath application of substance P (5.0 μM), a NK1R agonist, significantly increases lung burst frequency ($P < 0.001$). These results collectively support the identification of a bullfrog RRG, one that is closely associated with lung ventilation and potentially homologous to the mammalian preBötzinger complex.

[1]Davies B, Brundage C & Taylor B. (2008) μ-opioid and neurokinin-1 receptor immunofluorescence and involvement in the neuroventilation of bullfrogs. Prepared for submission to The Journal of Physiology.

1.1 Introduction

Two distinct neuronal networks are proposed as essential for respiratory rhythm generation in mammals (Onimaru *et al.*, 1997; Rekling & Feldman, 1998): inspiratory neurons in the preBötzinger Complex (PBC; Smith *et al.*, 1991; Gray *et al.*, 2001) and pre-inspiratory neurons rostral to the PBC in the parafacial respiratory group (pFRG; Onimaru *et al.*, 1995). Opiates slow breathing and inhibit the recruitment of inspiratory PBC neurons by pFRG neurons, but have no effect on pre-inspiratory pFRG neurons (Takeda *et al.*, 2001). Normal breathing is a rhythmic pattern produced by a neural network that includes the PBC and pFRG operating in concert with one another. Opiates can uncouple PBC activity from pFRG activity (Mellen *et al.*, 2003), and when isolated under special conditions, the PBC and pFRG are each capable of rhythm generation (Smith *et al.*, 1991; Guyenet et al., 1999). Thus, mammalian respiratory rhythm may be the output of two putative respiratory rhythm generators (RRG).

Post-metamorphic bullfrogs have two distinct breathing-related motor patterns: buccal ventilation, a remnant of gill ventilation (Winmill & Hedrick, 2003), and lung ventilation, which often occurs in episodes (Kinkead & Milsom, 1994). Buccal and lung ventilation are controlled by two distinct neuronal oscillators (Wilson *et al.*, 2002). In intact adult frogs and juvenile *in vitro* brainstem preparations, opiates depress lung burst frequency while buccal rhythm remains constant (Vasilakos *et al.*, 2005), suggesting similarities between the mammalian PBC and a putative lung RRG of bullfrogs.

The mammalian PBC contains a high density of neurokinin-1 receptor- (NK1R) expressing neurons (Gray *et al.*, 1999). These neurons are necessary for maintaining a normal respiratory rhythm (Gray *et al.*, 2001; Wang *et al.*, 2002) and are located in all chemosensory sites (Nattie & Prabhakar, 2001; Nattie & Li, 2002). The PBC also contains a large number of substance P (SP) immunoreactive neurons that synapse with approximately 1/3 of NK1R-expressing neuron dendrites (Liu *et al.*, 2004). NK1Rs are

the preferred binding site for SP (Makeham *et al.*, 2001), and local application of SP to the PBC increases respiratory frequency in rats (Gray *et al.*, 1999).

Colocalization of NK1R and μ-opioid receptors (μOR) has been used to determine the location of the PBC in mammalian brainstem slices (Gray *et al.*, 1999). The aim of this study was to use double-label immunofluorescence techniques to determine a location of the putative lung RRG based on NK1R and μOR colocalization and to determine whether similar areas of coexpression are present throughout tadpole development. I hypothesized that NK1R and μOR colocalization would mark the lung RRG of the bullfrog. Furthermore, I characterized the bullfrog response to SP, antagonist D, DAMGO and naloxone as a justification for interpreting colocalization of these receptors as an implication of an area involved in lung respiratory rhythm generation.

1.2 Methods

1.2.1 Animals

Electrophysiology studies were performed on *Lithobates* (formerly *Rana) catesbeiana* at two developmental stages, juvenile frogs (n = 12) and early-stage tadpoles (n = 12), of either sex purchased from a commercial supplier (Sullivan, Nashville, TN). All animals were maintained at 25 °C in aquaria and were fed crickets (frogs) or fish food (tadpoles). The University of Alaska Institutional Animal Care and Use Committee (IACUC) approved the animal and research protocols. Experimental protocols adhered to local and national ethics standards.

Tadpoles were used for immunofluorescence protocols to compare mean intensity and colocalization of NK1R and μOR among developmental stages. My classification of tadpoles, based on the developmental stages defined by Taylor and Kollros (1946), was as follows: early-stage animals were TK I-X (n = 6), middle-stage animals were XI-XVII

(n = 6), late-stage animals were XVIII-XXV (n = 6) and juvenile frogs were stage XXV+ (n = 8).

1.2.2 Materials

The neuromodulators DAMGO ([D-Ala^2, N-Me-Phe^4, Gly^5-ol]-Enkephalin acetate salt), antagonist D ([D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-Substance P), substance P acetate salt hydrate and naloxone hydrochloride dehydrate were all purchased from Sigma Chemical (St. Louis, MO). Secondary antibodies (anti-mouse rhodamine red and anti-rabbit fluoresceine) and donkey serum for immunofluorescence were obtained from Jackson Immunoresearch (West Grove, PA). The rabbit anti-μ-opioid receptor primary antibody was purchased from Immunostar (Hudson, WI). The mouse anti-NK1-receptor primary antibody was purchased from Invitrogen Corp. (Carlsbad, CA).

1.2.3 Surgical preparation

Each animal was anesthetized by immersion for 1-2 min in a cold (4 °C) 0.5 % wt/vol solution of tricaine methanesulfonate (MS222; Sigma Chemical, St. Louis, MO) in dechlorinated water buffered with NaOH to pH 7.8. The dorsal cranium was removed and the forebrain rostral to the optic lobes was resected. The fourth ventricle was exposed by removing the choroid plexus. The brainstem and spinal cord were removed *en bloc* from the cranium and spinal canal. The dura mater was stripped, and the brain was transected rostral to the optic tectum and caudal to the brachial nerve. During the dissection, the brainstem was superfused with cold (4 °C) artificial cerebrospinal fluid (aCSF) composed of (in mM) 104 NaCl, 4 KCl, 1.4 $MgCl_2$, 10 D-glucose, 25 $NaHCO_3$ and 2.4 $CaCl_2$.

The isolated brainstem was transferred to a low-volume (0.50 ml) flow-through plexiglas recording chamber. The isolated brainstem was supported ventral side up between coarse nylon mesh allowing every surface to be bathed with aCSF from rostral

to caudal regions at a rate of 5 ml·min^{-1}. Supply of aCSF, equilibrated with an O_2-CO_2 mixture to produce pH 7.8, flowed through plastic tubing to the chamber. After dissection, the brainstem was allowed to stabilize for 1 h at 25 °C, pH 7.8 and ~9 Torr Pco_2. During the experiments, pH of the aCSF was maintained by adjusting the concentrations of O_2 and CO_2. CO_2 was analyzed with a Datex 223 CO_2 Monitor (Puritan-Bennett Corp., Pleasanton, CA).

1.2.4 Nerve recording

Roots of the facial and hypoglossal nerves were drawn into glass suction electrodes pulled from 1-mm-diameter capillary glass to tip diameters of 30-60 μm. Whole-nerve discharge was amplified (100x by a DAM-50 amplifier, World Precision Instruments; Sarasota, FL; and then 1000x by model 1700 polarographic amplifier; A-M Systems, Carlsborg, WA), and filtered (100 Hz high-pass and 1 kHz low-pass by the second amplifier). The amplified and filtered output was sent to a data acquisition system (Powerlab, AD Instruments, Colorado Springs, CO), which sampled data at 1 kHz, integrated data (full-wave rectified and averaged over 200 ms), as well as recorded and archived whole-nerve discharge as neurograms.

The neurograms recorded from the facial and hypoglossal nerves consisted of rhythmic activity of two types: low amplitude, high frequency bursts representing buccal ventilation and high amplitude, low frequency bursts representing lung ventilation. The present investigation focused on lung neuroventilation. Thus, I investigated the role of NK1R and μOR on lung rhythm generation.

1.2.5 Protocol

Following initial dissection, preparations were allowed to stabilize for 1 h. Following stabilization, baseline nerve activity was recorded for 30 min, while the brainstem was superfused with aCSF equilibrated with 1.5 % CO_2, balance O_2 (pH 7.8). The brainstem

was treated for 30 min with an agonist, then for 30 min with an antagonist for the receptor of interest. The preparation then underwent a 30-min washout phase. For NK1R studies, preparations were superfused with 5.0 μM SP and 750 nM antagonist D (n = 7). For μOR studies, preparations were superfused with 100 nM DAMGO and 1.0 μM naloxone (n = 5). These concentrations were chosen based on Vasilakos *et al.* (2005), whose study compared the neuroventilatory effects of DAMGO in mammals and bullfrogs.

1.2.6 Immunofluorescence

Brainstems were isolated from 8 juvenile frogs and 6 early-, middle- and late-stage tadpoles according to the protocol described above (Surgical Preparation). Brainstems were fixed in 4 % paraformaldehyde in PBS for 24 h and stored in 30 % glucose until slicing. Brainstems were frozen in Optimum-Cutting-Temperature (OCT) compound (Fischer; Pittsburgh, PA). Brainstems were mounted and cross-sectioned using an IEC Minitome cryostat (IEC; Needham Heights, MA). 16-μm slices were mounted on gelatin-coated slides and stored at -80 °C until staining. Slides were washed for 3 10-min intervals in phosphate buffered saline (PBS) composed of (in mM) 136.9 NaCl, 2.68 KCl, 10.4 Na_2HPO_4 and 1.8 KH_2PO_4. Slides were incubated (4 °C) for 2 h with blocker (3 % Triton-X and 5 % Donkey Serum in PBS) and for 48 h with the primary antibody solution. The primary antibodies were mouse anti-NK1R (1:20,000) and rabbit anti-μOR (1:75,000). Slides were rinsed twice for 5 min and once for 1 h in PBS. Slides were incubated (4 °C) for 1 h with secondary antibody solution containing anti-mouse rhodamine red (RRX, 1:700) and anti-rabbit fluoresceine (FitC, 1:500). Slides were rinsed 6 times for 5 min with PBS and stored at 4 °C to dry. Slides were hydrated with Vectashield (Vector Laboratories, Inc., Burlingame, CA) and cover slipped.

1.2.7 Microscopy

Slices were viewed using an Axioplan Imaging fluorescent microscope (Zeiss, Thornwood, NY) equipped with Metamorph imaging software (Molecular Devices, Downington, PA). Locations of receptor colocalization were determined using measurement and calibration options in Metamorph.

1.2.8 Data analysis and statistics

Electrophysiology. Lung burst frequency was determined by counting the number of lung bursts per minute for every minute of each 30-min treatment. Analysis of lung neuroventilation was divided into 4 categories: baseline, agonist, antagonist and washout. Graphs were generated using SigmaPlot (Systat Software Inc., San Jose, CA) and statistical analyses were conducted with SigmaStat (Systat Software Inc., San Jose, CA). One-way repeated measures analysis of variance (RM-ANOVA) followed by a Student-Newman-Keuls multiple comparison test was used to determine statistical differences in mean frequencies of each 30-min treatment. All data are reported as mean ± S.E.M.

Immunofluorescence. Color was added to the individual channels using Image J software (online download), and colocalization images were generated by overlaying the individual channels with ColocalizerPro (Colocalization Research Software). Colocalization was quantified with 2 colocalization coefficients, Pearson's correlation coefficient and overlap coefficient according to Manders. The Pearson's correlation coefficient ranges from -1 to 1. A value of -1 indicates no correlation in fluorescence localization, and a value of 1 indicates a perfect correlation between the 2 channels. The overlap coefficient according to Manders ranges from 0 to 1. A value of 0 indicates that none of the pixels overlap, while a value of 0.7, for example, indicates that 70 % of the pixels overlap. Colocalization coefficients were calculated after background correction using ColocalizerPro. Mean intensity of the receptors was measured using Image J software.

1.3 Results

1.3.1 Effect of substance P on neuroventilatory output

Isolated brainstems of juvenile bullfrogs (n = 7) underwent 30 min of the following: baseline, 5.0 μM SP, 750 nM antagonist D and washout (Fig 1.1A). Bath application of SP increased lung burst frequency in juvenile bullfrog isolated brainstem preparations. Recordings from the facial nerve during baseline conditions illustrated a pattern of lung burst activity consisting of infrequent episodes of 2 or 3 bursts (Fig. 1.1B). Recordings from the same nerve during bath application of SP showed lung bursts occurring as frequent single bursts, with less organized episodes (Fig. 1.1C). Average lung frequency at baseline was 5.8 ± 0.2 bursts·min^{-1}, while frequency during SP exposure increased significantly to 10.3 ± 0.5 bursts·min^{-1} ($P < 0.001$, Fig. 1.1D). During subsequent bath application of antagonist D the lung frequency significantly decreased to 5.8 ± 0.2 bursts·min^{-1} ($P < 0.001$), and lung frequency returned to baseline levels during washout.

Isolated brainstems of early-stage tadpoles (n = 6) underwent the same treatments. SP significantly increased lung burst frequency from 1.4 ± 0.2 bursts·min^{-1} to 3.8 ± 0.3 bursts·min^{-1} ($P < 0.001$, Fig. 1.2). During subsequent exposure to antagonist D, lung burst frequency significantly decreased to 2.0 ± 0.2 bursts·min^{-1} ($P < 0.001$). Antagonist D did not return frequency to baseline ($P = 0.004$).

1.3.2 Effect of DAMGO on neuroventilatory output

Juvenile bullfrogs (n = 5) underwent 30 min of the following: baseline, 100 nM DAMGO, 1.0 μM naloxone and washout (Fig. 1.3A). Under baseline conditions, lung bursts occurred in infrequent clusters of 2-12 bursts per episode (Fig. 1.3B) while burst pattern during bath application of DAMGO treatment displayed infrequent single bursts or episodes of 2 lung bursts (Fig. 1.3C). Lung frequency during baseline was 7.5 ± 0.5 bursts·min^{-1} and significantly decreased to 4.2 ± 0.4 bursts·min^{-1} during DAMGO exposure ($P < 0.001$, Fig. 1.3D). During naloxone exposure lung frequency significantly

increased to 5.3 ± 0.4 burst·min^{-1} ($P = 0.001$), but did not restore frequency to baseline levels ($P < 0.001$).

Early-stage tadpoles (n = 6) underwent the same treatments. Lung burst frequency significantly decreased from 1.5 ± 0.1 bursts·min^{-1} to 1.1 ± 0.1 bursts·min^{-1} during DAMGO exposure ($P = 0.011$). Frequency subsequently increased to 2.9 ± 0.3 bursts·min^{-1} during naloxone exposure ($P < 0.001$), a level significantly greater than baseline frequency ($P < 0.001$, Fig. 1.4).

1.3.3 Colocalization of NK1R and μOR in juvenile bullfrogs

Cross-section slices of 8 juvenile bullfrog brainstems revealed a site of NK1R and μOR colocalization near the ventral surface of the medulla caudal to the facial nerve (Fig. 1.5). Additional areas of coexpression closer to the dorsal surface may indicate the facial motor nucleus and the auditory nucleus.

The approximate location of the primary colocalization, the region of interest, was measured consistently on the right half of each slice, reporting the region of interest distance from the top (390.7 ± 66.7 μm) and bottom (172.5 ± 26.6 μm) of the slice, from the midline (666.5 ± 26.6 μm) and from the right edge (503.5 ± 68.2 μm). The height and width of the ROI were calculated as 261.8 ± 8.9 μm and 235.9 ± 21.6 μm, respectively.

The level of colocalization was quantified based on 2 colocalization coefficients. Colocalization coefficients for the region of interest were computed for each of the 8 juvenile bullfrog brainstems. The Pearson's correlation coefficient (Rr) was 0.8 ± 0.03 and the overlap coefficient according to Manders (R) was 0.9 ± 0.02.

1.3.4 Ontogeny of NK1R and μOR colocalization

Pearson's correlation coefficient and overlap coefficient according to Manders were measured for early-, middle- and late-stage tadpoles (n=6 for each group). The mean Pearson's coefficients for early-, middle- and late-stage tadpoles were 0.8 ± 0.03, 0.8 ± 0.03 and 0.7 ± 0.03, respectively. There was no significant difference in the Pearson's correlation coefficient among developmental stages. Overlap coefficient according to Manders for early-, middle- and late-stage tadpoles were 0.9 ± 0.02, 0.9 ± 0.01 and 0.9 ± 0.02, respectively. There was a significant difference between middle- and late-stage tadpoles ($P < 0.05$) and between middle-stage tadpoles and juvenile frogs ($P < 0.05$). Cross-section slices of early-, middle- and late-stage tadpoles did not reveal a defined area of receptor colocalization (Fig. 1.6) like that expressed in juvenile frogs (Fig. 1.5).

1.3.5 Staining intensity of NK1R and μOR

For 6 early-, middle-, and late-stage tadpoles and 8 juvenile frogs, the mean intensity for NK1R immunofluorescence in the region of interest was 646.9 ± 74.1, 505.2 ± 34.7, 1106.2 ± 154.1 and 787.7 ± 38.9, respectively (Fig. 1.7). The intensity of fluorescence based on NK1R- and μOR-antibody binding was significantly different in the brainstems of middle-stage tadpoles compared to that of late-stage tadpoles ($P < 0.05$). All other groupings of tadpole and frog stages comprised statistically homogenous subsets.

For 6 early-, middle- and late-stage tadpoles and 8 juvenile frogs, the mean intensities for μOR immunofluorescence in the region of interest were 292.6 ± 39.1, 300.6 ± 26.6, 391.4 ± 60.7 and 612.4 ± 33.2, respectively (Fig. 1.8). There was a significant difference in receptor intensity between juvenile frogs and all tadpole stages.

1.4 Discussion

The bullfrog neuroventilatory response to substance P (SP) and DAMGO is consistent throughout development with SP eliciting an increase in lung burst frequency

whereas DAMGO depresses lung burst frequency. Based on double-label immunofluorescence, I propose a location of a bullfrog respiratory rhythm generator (RRG) that is closely associated with lung ventilation and is potentially homologous to the mammalian preBötzinger complex (PBC) RRG. As such, my findings support the location of a lung RRG proposed by Wilson *et al.* (2002) and the homology proposed by Vasilakos *et al.* (2005) and Wilson *et al.* (2006).

1.4.1 Opiate-induced neuroventilatory depression

The neuroventilatory bursts indicate that buccal and lung breaths are timed to one another in the isolated juvenile bullfrog brainstem preparation (Wilson *et al.*, 2002). One site, located between the auditory and glossopharyngeal nerves is essential for lung bursts but not buccal bursts, and it has been called the lung area (Wilson *et al.*, 2002). Another site, at the level of the vagus nerve, is essential for buccal bursts but not lung bursts (Wilson *et al.*, 2002), and it is called the buccal area. Treatment with opiates is one means to uncouple the respiratory oscillators of mammals (Mellen *et al.*, 2003) and bullfrogs (Vasilakos *et al.*, 2005). In late-stage tadpoles, lung frequency increases with CO_2 (Torgerson *et al.*, 1997), while buccal bursts are dependent on $GABA_A$ (Galante *et al.*, 1996) and are not responsive to CO_2 (Taylor *et al.*, 2003). Buccal and lung bursts also differ in their response to 5-HT (Belzile *et al.*, 2002) and $GABA_B$ receptor agonists (Straus *et al.*, 2000; Belzile *et al.*, 2002). Thus, despite being timed to one another, there is evidence to suggest that buccal and lung bursts are controlled in different areas of the brainstem and are differently influenced by a variety of chemicals.

Bath application of the μOR agonist DAMGO to bullfrog brainstem preparations *in vitro* results in a significant decrease in lung neruoventilation, suggesting that μOR have a role in modulating lung respiratory rhythm. These results are consistent with those of Vasilakos *et al.* (2005) who showed that the overall frequency of lung bursts decreased with application of DAMGO. These authors found that buccal burst frequency remained

constant in both stages of animals during DAMGO application. In intact frogs, morphine injection significantly decreased lung ventilation, but the total number of ventilatory events (buccal plus lung) remained constant (Vasilakos *et al.*, 2005); therefore, buccal ventilation replaced lung ventilation under the influence of DAMGO. Data from my study confirm the depression in lung ventilation with DAMGO treatment and show recovery from the depressive effects during subsequent treatment with naloxone.

Mammalian treatment with opiates produced similar results. Following application of DAMGO, rat respiration gradually slowed, and returned to control values during application of naloxone (Mellen *et al.*, 2003). Quantal slowing, which is slowing by omitting events, during opiate exposure in mammals suggests a dual network for respiratory rhythm generation. Quantal slowing is apparent as separate peaks at integer multiples of the control period (Mellen *et al.*, 2003). Results from that study led to the conclusion that PBC inspiratory neurons are driven by pre-inspiratory neurons, to generate respiratory rhythm.

Opiate-induced quantal slowing of frog respiration suggests a similar respiratory control network. The occurrence of lung bursts is reduced by DAMGO while the buccal burst activity is unchanged. Homologies between the PBC and lung RRG have been proposed based on these findings (Vasilakos *et al.*, 2005). The power stroke for lung inflation is produced by the putative lung oscillator in amphibians (Wilson *et al.*, 2006) and the PBC in mammals (Ramirez *et al.*, 1998; Mellen *et al.*, 2003; Janczewski & Feldman, 2006), supporting the homology hypothesis.

1.4.2 Neuroventilatory responses to substance P

The ventilatory response to SP is well documented in mammals. SP modulates activity of the rhythm generator in newborn rats (Ptak *et al.*, 1999), and changes in the level of SP in the nucleus tractus solitarius have suggested a role for SP in regulating the

onset of breathing (Srinivasan *et al.*, 1991). *In vitro* brainstem preparations of the newborn rat exhibit increased respiratory frequency with SP treatment (Yamamoto *et al.*, 1992). Blockade of SP receptors with spantide results in significant irregular breathing activity and decreased frequency in mice (Telgkamp *et al.*, 2002), suggesting a role of endogenous SP in the regularity and frequency of eupneic respiration.

The significant increase in lung burst frequency with bath application of SP to juvenile bullfrog and tadpole brainstem preparations *in vitro* suggests that NK1Rs play a role in regulating respiratory rhythm throughout development. Thus, SP has similar influences through anuran and mammalian ontogeny. Injection of SP conjugated with saporin, which specifically destroys NK1R-expressing neurons (Mantyh *et al.*, 1995; Mantyh *et al.*, 1997), into the PBC of adult rats produces abnormal breathing periods (Gray *et al.*, 2001; Wang *et al.*, 2002). Given the established similarities between mammalian and amphibian neuronal networks, along with results from this study, it is possible that lesions of NK1Rs in my putative lung RRG, which I propose is the same as the lung area proposed by Wilson *et al.* (2002), would also produce abnormal breathing periods.

1.4.3 Immunofluorescence of the putative lung RRG

High densities of NK1Rs within the PBC (Gray *et al.*, 1999) are used to locate the PBC on mammalian brainstem slices. The PBC NK1Rs colocalize with μORs (Gray *et al.*, 1999). Similar respiratory responses of bullfrogs and mammals to SP and DAMGO suggest that colocalization of these receptors will mark the putative lung RRG. Lack of buccal response to DAMGO (Vasilakos *et al.*, 2005) suggests that colocalization of these receptors will not mark the putative buccal RRG in brainstem slices.

One distinct site of colocalization was found caudal to the facial nerve root, and is located near the bullfrog facial nucleus, an area previously linked with bullfrog

respiratory rhythm generation (Wilson *et al.*, 2002). Nano-injection of DAMGO and SP into the proposed site of the lung RRG would give further evidence that the colocalized area marks the putative lung RRG in bullfrogs.

Lack of receptor coexpression in developing tadpole brainstems suggests that during development there is a change in receptor expression within the putative lung RRG. Throughout development, the NK1R intensity in the region of interest does not display an increasing or decreasing trend. This suggests that NK1Rs within the putative lung RRG are present in all stages of development. However, μOR intensity in the region of interest increases at metamorphosis. The lack of receptor colocalization during development may be attributable to the lack of μOR expression in tadpoles. The receptor-staining intensity in juvenile frogs is significantly greater than in late-stage tadpoles, suggesting tadpoles in late-stage development, while being more dependent on lung ventilation than at earlier stages, do not have the same receptor composition within the RRG as bullfrogs do after metamorphosis. Despite the lack of μORs in the putative lung RRG of tadpoles, lung neuroventilation is still responsive to DAMGO. This suggests the existence of another site important to lung neuroventilation, one which is sensitive to opiates.

The fact that early-stage tadpoles respond like juvenile frogs to bath application of SP and DAMGO shows that the NK1Rs and μORs are present within the brainstem, but does not indicate the relative location of these receptors. Nano-injection into various sites would help reveal the location of respiratory-related receptors throughout development.

1.5 Acknowledgements

This work was supported by NIH-NINDS 2U54NS041069-06AI. I would like to thank Dr. Barbara Taylor for serving as my graduate advisor, as well as Dr. Michael Harris and Dr. Marina Castillo, who served on my graduate committee. Thanks to Cord Brundage for his help with the immunofluorescence and tadpole/frog dissection.

Additional thanks to the University of Alaska Fairbanks, Department of Biology and Wildlife and the Institute of Arctic Biology for their support. I would also like to thank the Specialized Neuroscience Research Program (SNRP) and the Alaska Basic Neuroscience Program.

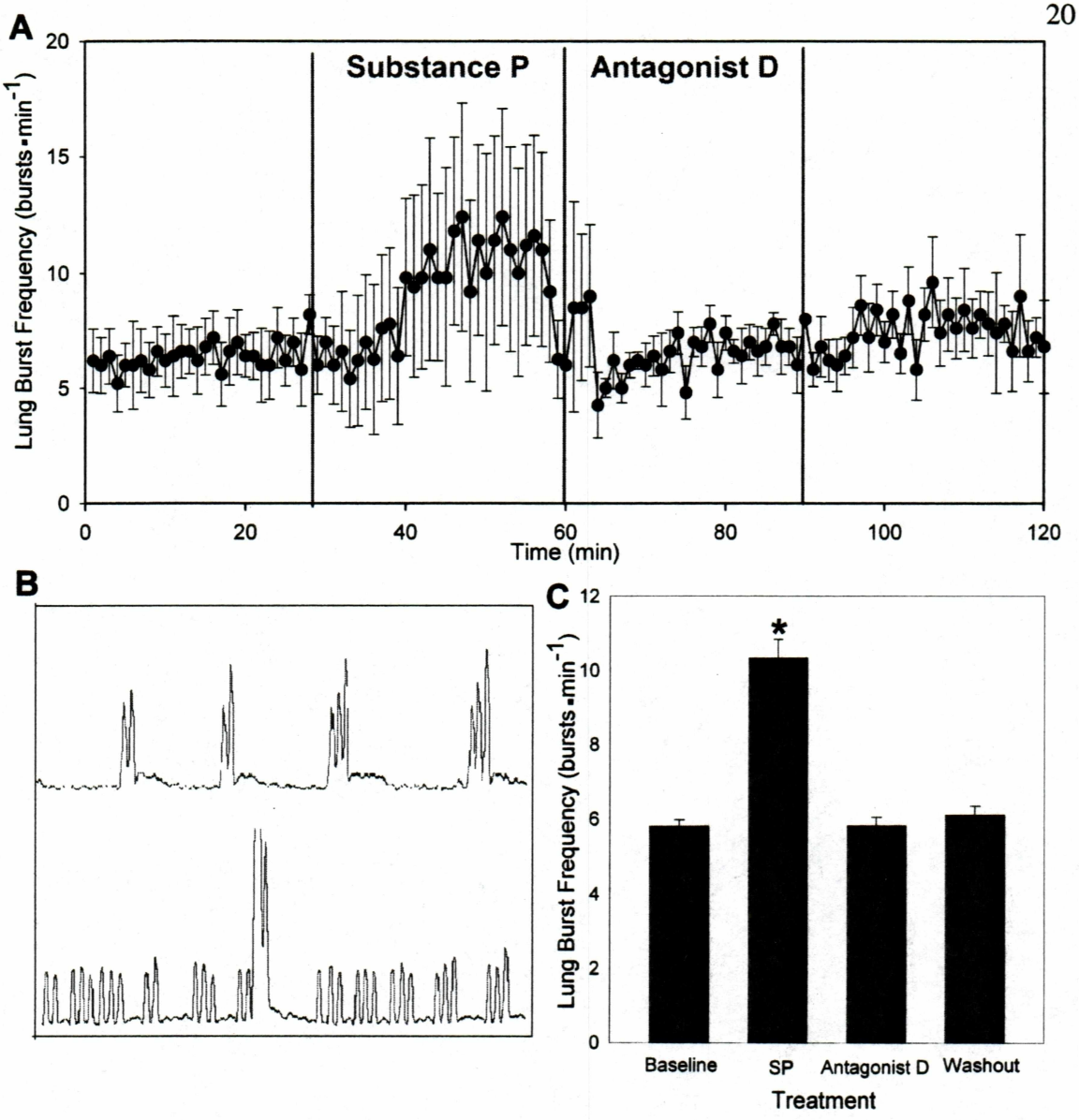

Figure 1-1. Juvenile bullfrog neuroventilatory response to bath application of SP and antagonist D. (A) Bath application of SP for 30 min increases lung burst frequency, which subsequently decreases during application of antagonist D. The 30-min washout period returns lung neuroventilation to baseline levels. (B) A 1-min section of a neurogram during baseline (top) and SP (bottom) from the same animal is shown. The lung neuroventilatory pattern during baseline consists of infrequent clusters of 2-3 bursts per cluster. Lung neuroventilation during SP displays frequent bursts with a less regular clustering of episodes. (C) Mean ± S.E.M. lung burst frequency for the 4 periods (baseline, SP, antagonist D, washout) for 7 preparations. Asterisk indicates a significant difference from all other treatments ($P < 0.05$).

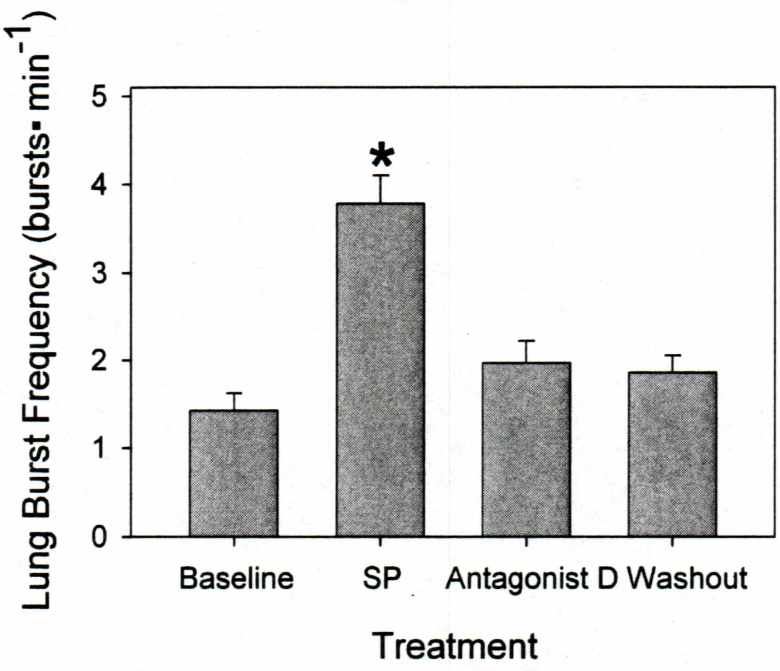

Figure 1-2. Early-stage tadpole neuroventilatory response to SP and antagonist D. SP significantly increases lung burst frequency, which subsequently decreased during antagonist D exposure (n = 6). Asterisk indicates a significant difference from all other treatments ($P < 0.05$).

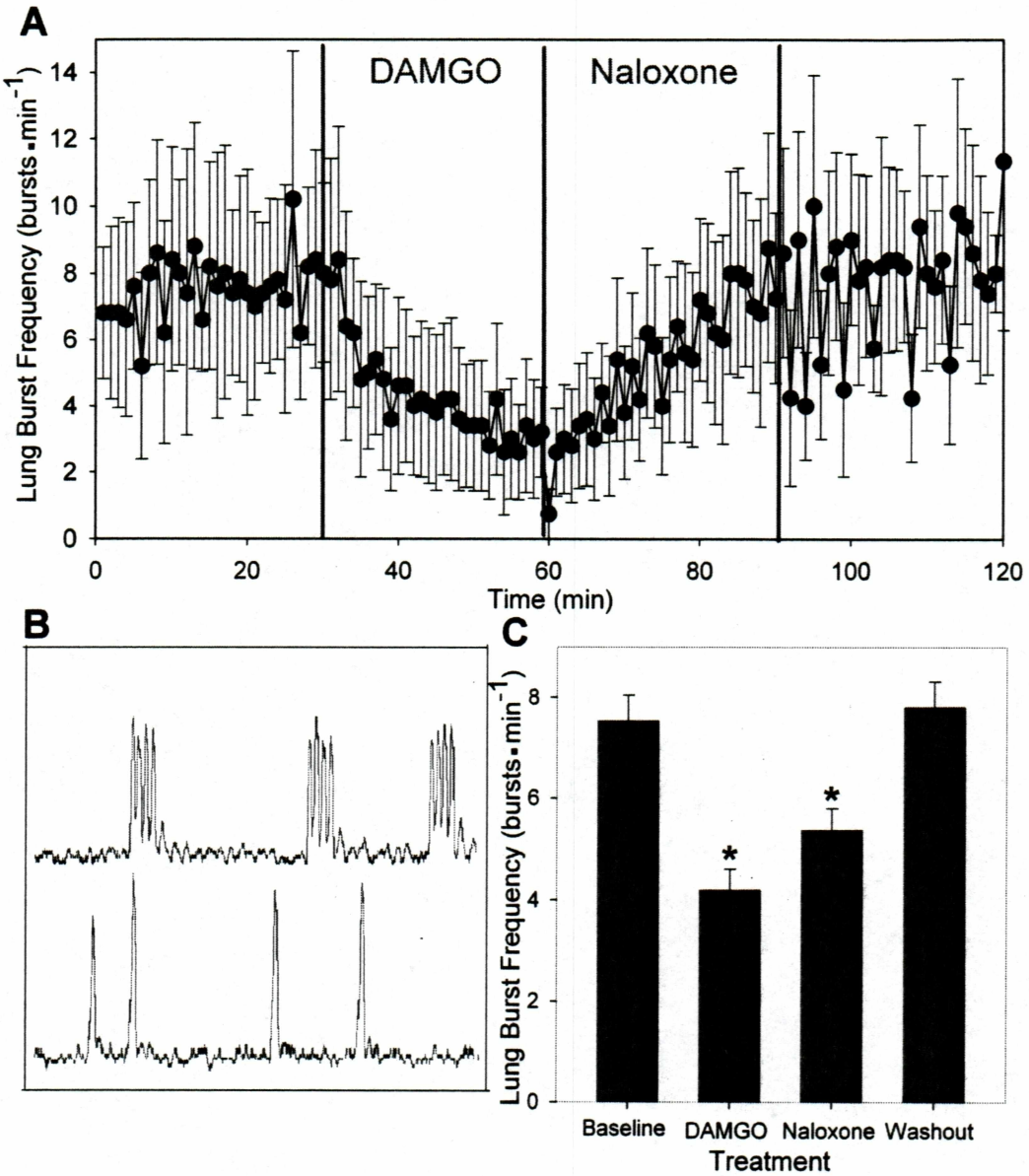

Figure 1-3. Juvenile bullfrog neuroventilatory response to bath application of DAMGO and naloxone. (A) Application of DAMGO for 30 min following baseline significantly reduced lung burst frequency. During subsequent treatment with naloxone for 30 min, lung burst frequency increased to near baseline levels. Washout returned lung burst frequency to baseline. (B) During baseline (top), lung burst pattern was infrequent clusters with 4 bursts per cluster. During DAMGO application (bottom), lung burst pattern was infrequent single bursts. Traces are from the same preparation. (C) Mean ± S.E.M lung burst frequency for the 4 periods (baseline, DAMGO, naloxone and washout) for 5 preparations. Asterisk indicates a significant difference from baseline and washout ($P < 0.05$).

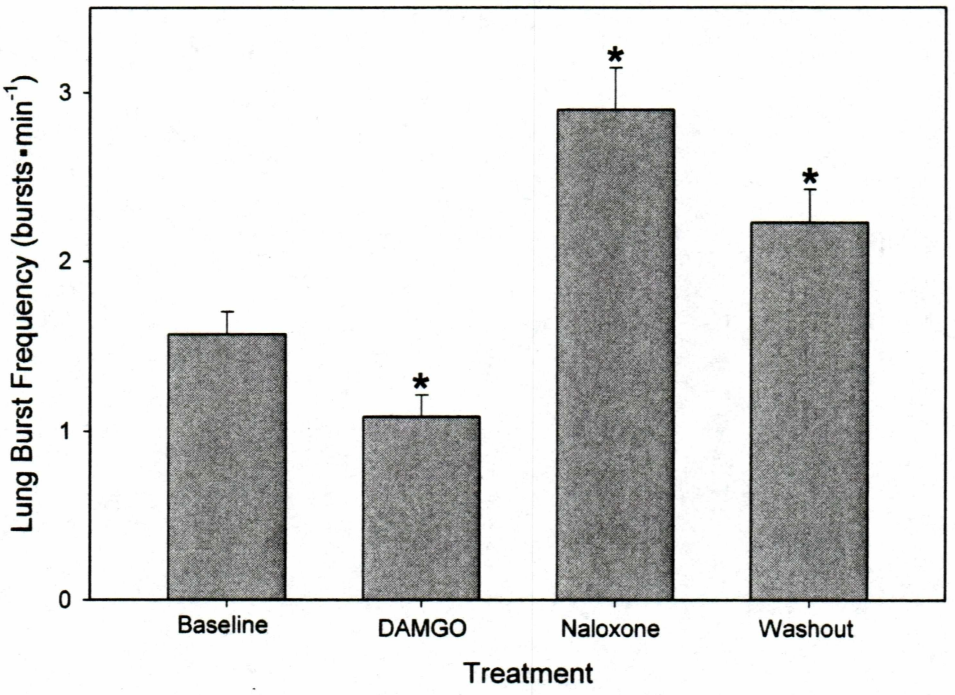

Figure 1-4. Early-stage tadpole neuroventilatory response to DAMGO and naloxone. DAMGO significantly reduced lung burst frequency, which subsequently increased beyond baseline levels with naloxone application (n = 6). Asterisks indicate a significant difference from all other treatments ($P < 0.05$).

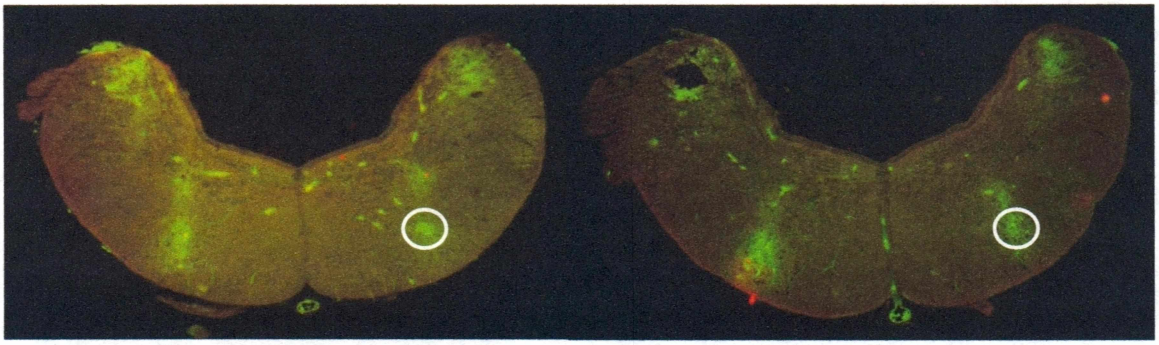

Figure 1-5. Immunofluorescence of NK1R amd μOR reveals distinct colocalization in juvenile bullfrog brainstems. Images are representative of two adult brainstems. The site of receptor colocalization in the juvenile brainstem, indicated by the circle, is consistent among all animals (n = 8).

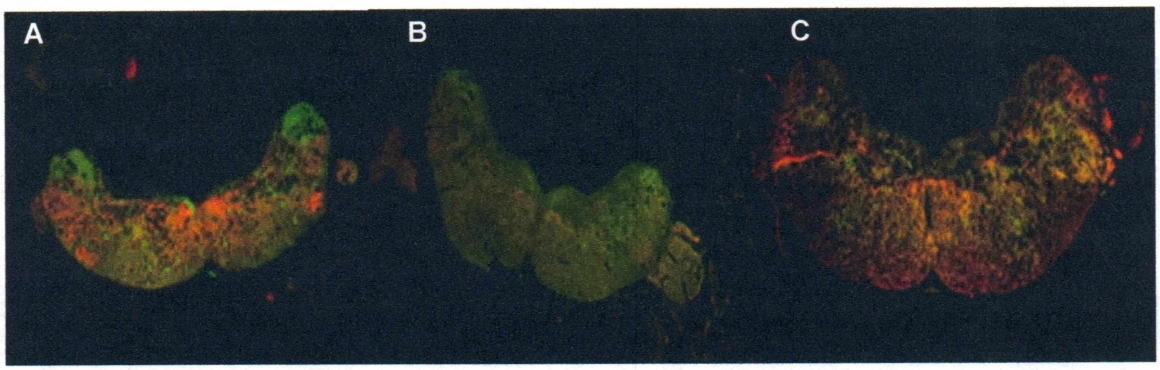

Figure 1-6. Ontogeny of NK1R and uOR colocalization. (A) Early-stage tadpole, (B) middle-stage tadpole and (C) late-stage tadpole cross-sections. There is a lack of any distinct site(s) of receptor colocalization during tadpole development.

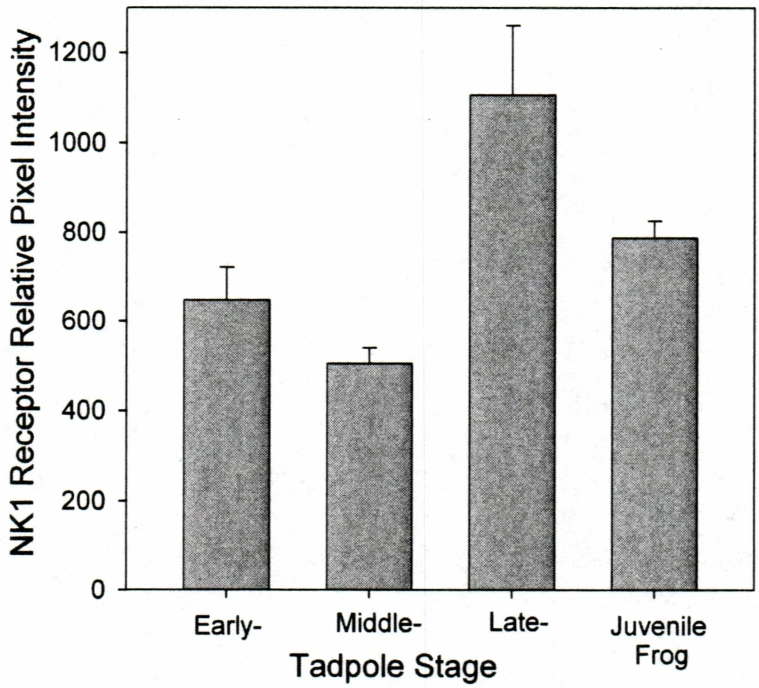

Figure 1-7. Mean intensity of NK1R immunofluorescence among developmental stages. Expression of NK1R, as indicated by antibody binding, in middle-stage tadpoles was significantly lower than that in late-stage tadpoles and juvenile frogs (n = 4 - 6).

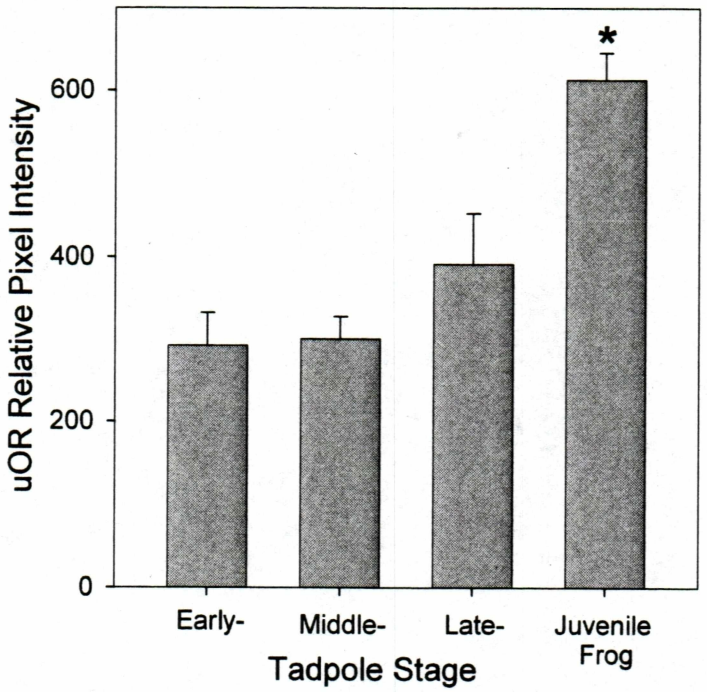

Figure 1-8. Mean intensity of μOR immunofluorescence among developmental stages. Expression of μ-opioid receptors, as indicated by antibody binding, in juvenile frogs was significantly higher than that in tadpoles of any developmental stage (n = 4 - 6). Asterisk indicates a significant difference from all other groups ($P < 0.05$).

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Chapter 2:

Involvement of substance P and DAMGO in lung pattern formation in bullfrogs[1]

Abstract

Neurokinin-1 (NK1R) and μ-opioid (μOR) receptors are colocalized in a region thought to be the lung respiratory rhythm generator (RRG) in juvenile bullfrogs. Activation of these receptors not only influences respiratory rhythm generation, but alters pattern formation as well. Substance P (SP; NK1R agonist), antagonist D (NK1R antagonist and the μOR agonist DAMGO decreased the number of lung bursts per episode ($P < 0.05$). Along with changes in episode size, all neuromodulators significantly influenced lung burst parameters including duration, amplitude and area. Lung burst cycle frequency (BCF) increased with SP and decreased with antagonist D ($P < 0.05$). DAMGO decreased BCF, which then increased during naloxone treatment ($P < 0.05$). SP increased episode frequency ($P < 0.05$), which then decreased during antagonist D treatment ($P< 0.05$). DAMGO decreased episode frequency ($P < 0.05$). These results collectively support the idea that pattern formation is an intrinsic property of the respiratory control network potentially controlled by a site other than the RRG.

[1]Davies B, Brundage C & Taylor B. (2008) Involvement of substance P and DAMGO in lung pattern formation in bullfrogs. Prepared for submission to The Journal of Physiology.

2.1 Introduction

Episodic ventilation is characteristic of many ectothermic vertebrates (Milsom, 1991), and some mammals in states of metabolic depression breath episodically (McArthur & Milsom, 1991a, 1991b; Castellini *et al.*, 1994) Breathing converts from continuous to episodic pattern in phocid seals during sleep and while diving (Castellini *et al.*, 1994; Milsom *et al.*, 1996). In many species of small mammals, breathing becomes episodic during hibernation (Malan, 1982). Episodic breathing is evident in newborn infants (Levine *et al.*, 2000) as well as the neonatal rat *in vitro* brainstem preparation (Mellen *et al.*, 2002) and rat pups in response to hypothermic conditions (Tattersall & Milsom, 2003). Episodic patterns of ventilaton occur when clusters of breaths are followed by non-ventilatory periods; the breathing episodes vary in numbers of individual breaths (Kinkead & Milsom, 1994, 1996, 1997). There is further complexity in this pattern in that variation can also occur in the duration of episodes and the duration of the non-ventilatory periods. Brainstem-spinal cord preparations of bullfrogs (Kinkead *et al.*, 1994), turtles (Douse & Mitchell, 1990) and neonatal rats (Hilaire *et al.*, 1989) exhibit episodic ventilatory pattern.

Studies examining the mechanisms underlying episodic breathing have involved analysis of ventilatory responses in animals exposed to a variety of stimuli including changes in respiratory gases as well as application of neurotransmitters, neuromodulators and drugs. Studies have looked at central and peripheral chemoreceptors (West *et al.*, 1987; Smatresk & Smits, 1991; Kinkead & Milsom, 1994), pulmonary stretch receptors (Kinkead & Milsom, 1996, 1997) and olfactory receptors (Kinkead & Milsom, 1996). Each receptor group influences breathing pattern by altering the number of breaths per episode, the duration of the non-ventilatory period between episodes, or both. However, none of the receptor groups targeted by these studies was identified as being exclusively responsible for episode formation. In general, treatments that alter episode formation also alter overall ventilation. Changes in ventilation occur through changes in breathing pattern, including the pattern of episodes. Thus, influences on episode formation, *per se*,

are difficult to distinguish from changes in episodes that are secondary to changes in ventilatory drive (for review see Kinkead, 1997). Two studies, however, have suggested that episode formation can be regulated independently from ventilation by nitric oxide (Harris *et al.*, 2002) and $GABA_B$ pathways (Straus *et al.*, 2000). It has been proposed that the mechanisms responsible for pattern formation may be an intrinsic property of the central respiratory control network (Kinkead *et al.*, 1994), an idea that supports the hypothesis that, in intermittent breathers, the episode is the fundamental output of the control network rather than the individual breath within the episode (Jackson, 1978).

Transecting the brainstem between the optic lobes and cerebellum eliminates breathing episodes consisting of more than 1 breath in the bullfrog, and the breathing pattern appears to consist of evenly spaced breaths (Oka, 1958a, 1958b) indicating that episode formation is under the influence of a site independent of the one responsible for producing respiratory rhythm. In frogs (Kinkead & Milsom, 1995) and toads (Gargaglioni & Branco, 2001), the nucleus isthmi (NI) has been proposed as the site responsible for pattern formation. The NI, located on the roof of the midbrain and base of the cerebellum, is responsible for relaying visual information between the lobes of the optic tectum (Udin, 1987). The NI goes through cellular rearrangement during metamorphosis (Senn, 1972), a period associated with the onset of episodic breathing (Burggren & Infantino, 1994). Lesions to the NI transformed the breathing pattern from episodes to evenly spaced breaths (Kinkead & Milsom, 1995), and the change was associated with a reduction in breathing frequency rather than a change in the distribution of breaths. This led to the conclusion that the NI is probably not directly responsible for turning breathing episodes on and off but provides input into respiratory network (Kinkead *et al.*, 1997).

Studies using brainstem-spinal cord preparations of bullfrogs (Kinkead *et al.*, 1994), turtles (Douse & Mitchell, 1990) and neonatal rats (Hilaire *et al.*, 1989) have

demonstrated the occurrence of episodic breathing *in vitro* despite the lack of ascending sensory afferents and descending inputs from higher centers, indicating that production of episodes is an intrinsic property of the respiratory control system.

Overall, determining the mechanisms responsible for episodic pattern formation has proven elusive. The goal of this study was to further investigate the types of receptors involved in pattern formation. Neurokinin-1 (NK1R) and μ-opioid (μORs) receptors have been shown to colocalize in a region near the facial nucleus in juvenile bullfrogs, and neuromodulators that act on these receptors influence putative lung ventilatory frequency (Davies *et al.*, 2008). This study characterizes the effects of substance P (SP), antagonist D, DAMGO and naloxone on the neuroventilatory pattern of juvenile bullfrogs and tests the hypothesis that the mechanisms responsible for pattern formation are an intrinsic property of the respiratory control network and may arise from an area independent of the RRG.

2.2 Methods

2.2.1 Animals

Studies were performed on *Lithobates* (formerly *Rana) catesbeiana* juvenile frogs (n = 12) of either sex, purchased from a commercial supplier (Sullivan, Nashville, TN). Classification of frogs was based on the scheme of Taylor and Kollros (1946); juvenile frogs were stage 25+. All animals were maintained at 25 °C in aquaria and were fed crickets. The University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) approved the animal and research protocols. Experimental protocols adhered to local and national ethics standards.

2.2.2 Materials

The neuromodulators DAMGO ([D-Ala^2, N-Me-Phe^4, Gly^5-ol]-Enkephalin acetate salt), antagonist D ([D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-SP), Substance P (SP) acetate salt

hydrate and naloxone hydrochloride dehydrate were all purchased from Sigma Chemical (St. Louis, MO).

2.2.3 Surgical preparation

Each animal was anesthetized by immersion for 1-2 min in a cold (4 °C) 0.5 % wt/vol solution of tricaine methanesulfonate (MS222; Sigma Chemical; St. Louis, MO) in dechlorinated water buffered with NaOH to pH 7.8. The dorsal cranium was removed and the forebrain rostral to the optic lobes was resected. The fourth ventricle was exposed by removing the choroid plexus. The brainstem and spinal cord were removed *en bloc* from the cranium and spinal canal. The dura mater was stripped, and the brain was transected rostral to the optic tectum and caudal to the brachial nerve. During the dissection, the brainstem was superfused with cold (4 °C) artificial cerebrospinal fluid (aCSF) composed of (in mM) 104 NaCl, 4 KCl, 1.4 $MgCl_2$, 10 D-glucose, 25 $NaHCO_3$ and 2.4 $CaCl_2$.

The isolated brainstem was transferred to a low-volume (0.50 ml) flow-through plexiglas recording chamber. The isolated brainstem was supported ventral side up between coarse nylon mesh allowing every surface to be bathed with aCSF from rostral to caudal regions at a rate of 5 $ml \cdot min^{-1}$. Supply of aCSF, equilibrated with an O_2-CO_2 mixture to produce pH 7.8, flowed through plastic tubing to the chamber. After dissection, the brainstem was allowed to stabilize for 1 h at 25 °C, pH 7.8 and ~9 Torr Pco_2. During the experiments, pH of the aCSF was maintained by adjusting the concentrations of O_2 and CO_2. CO_2 was analyzed with a Datex 223 CO_2 Monitor (Puritan-Bennett Corp., Pleasanton, CA).

2.2.4 Nerve recording

Roots of the facial and hypoglossal nerves were drawn into glass suction electrodes pulled from 1-mm-diameter capillary glass to tip diameters of 30-60 μm. Whole-nerve discharge was amplified (100x by a DAM-50 amplifier, World Precision Instruments; Sarasota, FL; and then 1000x by model 1700 polarographic amplifier, A-M Systems; Carlsborg, WA) and filtered (100 Hz high-pass and 1 kHz low-pass by the second amplifier). The amplified and filtered output was sent to a data acquisition system (Powerlab, AD Instruments; Colorado Springs, CO), which sampled data at 1 kHz, integrated data (full-wave rectified and averaged over 200 ms), as well as recorded and archived the whole-nerve discharges as neurograms.

The neurograms recorded from the facial and hypoglossal nerves consisted of rhythmic activity of two types: low amplitude, high frequency bursts representing buccal ventilation and high amplitude, low frequency bursts representing lung ventilation. The present investigation focused on lung ventilation, which exhibits a rhythmic, episodic pattern. Thus, here I investigated the effects of SP, antagonist D, DAMGO and naloxone on this rhythmic and episodic pattern.

2.2.5 Protocol

Following stabilization, baseline nerve activity was recorded for 30 min during which the brainstem was superfused with aCSF at pH 7.8 equilibrated with 1.5 % CO_2 balance O_2. The brainstem was treated for 30 min with an agonist followed by 30 min with an antagonist for the receptor of interest. The preparation then experienced a 30-min washout period. For NK1R studies, preparations were treated with 5.0 μM SP and 750 nM antagonist D (n = 7). For μOR studies, preparations were exposed to 100 nM DAMGO and 1.0 μM naloxone (n = 5).

2.2.6 Data analysis and statistics

For each 30-min treatment, the mean number of lung bursts per episode was calculated. The percentage of episodes containing various numbers of lung bursts were calculated and compared among treatment groups. Episode frequency for each 30-min treatment was calculated by dividing the number of episodes by 30 min. Breath cycle frequency (BCF) within episodes was calculated by determining the inverse of the duration from the beginning of one lung burst to the beginning of a subsequent lung burst multiplied by 60. Other burst parameters including lung burst duration, amplitude and area were also calculated. BCF and burst parameters were calculated for a 3-min period 20 min into the SP treatment and 25 min into all other treatments. One-way repeated measures analysis of variance (RM-ANOVA) followed by the Student-Newman-Keuls multiple comparison test was used to compare data collected during the various treatments. All data are represented as mean ± SEM.

2.3 Results

2.3.1 SP and DAMGO influence the number of breaths per episode

SP and antagonist D both significantly decreased the number of lung bursts per episode from 1.6 ± 0.03 to 1.5 ± 0.02 and 1.3 ± 0.03, respectively ($P < 0.05$, Fig. 2.1A). Baseline, SP and antagonist D treatments each had the highest percentage of episodes containing single bursts (Fig. 2.1C). The percentage of single-burst episodes increased from 34 % to 47 % to 79 % during baseline, SP and antagonist D treatment, respectively. The slope of the line for percentages of episodes containing greater numbers of lung bursts decreased at a faster rate for baseline and antagonist D exposure than for SP treatment (Fig. 2.1E). Interestingly, antagonist D treatments contained the greatest percentage of episodes that had 4 or more bursts while baseline had the lowest percentage.

Baseline activity during opiate experiments had a mean number of lung bursts per episode of 2.7 ± 0.1 (Fig. 2.1B). This value decreased significantly during DAMGO exposure to 2.2 ± 0.1 ($P < 0.001$). Lung bursts per episode rose to 2.6 ± 0.1 during naloxone treatment, though this value was significantly lower than baseline ($P < 0.001$). Preparations during all treatments had the highest percentage of episodes containing 2 lung bursts (Fig. 2.1D). The percentage of episodes with two bursts decreased from 47 % during baseline to 43 % during DAMGO and 31 % during naloxone. The percentage of episodes containing higher numbers of lung bursts was lower during DAMGO exposure (Fig. 2.1F). The slope of the line during naloxone treatment decreased faster than baseline levels, but the decrease did not drop as low as with DAMGO exposure.

2.3.2 Lung burst parameters and BCF are affected by SP and DAMGO

SP decreased lung burst duration from 1.1 ± 0.1 sec to 0.9 ± 0.02 sec ($P = 0.005$, Fig. 2.2A). During antagonist D application lung burst duration significantly increased to 1.2 ± 0.9 sec ($P < 0.001$), which was equivalent to duration during baseline. Lung burst amplitude during baseline was 0.05 ± 0.003 V and significantly increased to 0.07 ± 0.002 V ($P < 0.001$) during SP application and remained significantly higher than baseline during antagonist D treatment (0.07 ± 0.004 V, Fig. 2.2C). Lung burst area increased significantly from a baseline value of 0.02 ± 0.001 V*sec to 0.02 ± 0.001 V*sec during SP ($P < 0.001$, Fig. 2.2E), and it increased, again significantly, during antagonist D treatment (0.03 ± 0.003 V*sec, $P < 0.001$).

DAMGO application significantly increased lung burst duration from 0.8 ± 0.01 sec during baseline to 0.9 ± 0.2 sec ($P < 0.001$, Fig. 2.2B). Duration decreased significantly to 0.6 ± 0.01 sec during naloxone application ($P < 0.001$). Lung burst amplitude also increased significantly from 0.04 ± 0.002 V to 0.06 ± 0.003 V during DAMGO ($P < 0.05$) and decreased to 0.05 ± 0.002 V ($P < 0.05$, Fig. 2.2D) during naloxone. Lung burst area reached is greatest values during DAMGO application with a mean area of 0.02 ±

0.001 V*sec, a significant increase from baseline areas of 0.01 ± 0.001 V*sec ($P < 0.05$, Fig. 2.2F). The lowest value of lung burst area was during naloxone, which decreased to 0.01 ± 0.001 V*sec.

BCF increased significantly during SP treatment from 25.3 ± 2.7 to 29.2 ± 1.5 bursts·min^{-1} ($P < 0.05$, Fig. 2.3A). BCF decreased significantly during antagonist D treatment to 13.8 ± 1.9 bursts·min^{-1} ($P < 0.05$). A significant decrease in BCF was also observed during DAMGO exposure, a decrease to 25.3 ± 3.4 bursts·min^{-1} from a baseline of 42.8 ± 2.8 bursts·min^{-1} ($P < 0.05$, Fig. 2.3B). Subsequently, during application of naloxone, BCF increased significantly to 51.2 ± 3.7 bursts·min^{-1} ($P < 0.001$).

2.3.3 Episode frequency is altered with exposure to SP and DAMGO

Episode frequency (episodes·min^{-1}) calculated for each 30-min treatment period showed a significant increase for SP application followed by a significant decrease during antagonist D application (Fig. 2.4A). Episode frequency increased from 3.7 ± 0.1 episodes·min^{-1} during baseline to 7.1 ± 0.3 episodes·min^{-1} during SP application ($P < 0.05$, Fig. 2.4B). Episode frequency decreased during antagonist D treatment to 4.5 ± 0.2 episodes·min^{-1} ($P < 0.05$), which was equivalent to baseline.

A decrease in episode frequency was apparent during DAMGO application with a less obvious increase during naloxone application (Fig. 2.5A). DAMGO significantly decreased episode frequency from 2.6 ± 0.1 to 1.9 ± 0.1 episodes·min^{-1} ($P < 0.05$, Fig. 2.5B). Naloxone slightly raised episode frequency to 2.0 ± 0.1 episodes·min^{-1}, though this value differed significantly from baseline values ($P < 0.05$).

2.4 Discussion

Overall, this study has shown that NK1R and μOR neuromodulators influence the ventilatory pattern in bullfrogs. SP, antagonist D, DAMGO and naloxone influence the number of lung bursts present in each episode, lung burst parameters and periodicity, as well as the frequency and periodicity of episodes.

2.4.1 Lung bursts per episode and episode frequency

Episodes can consist of any number of lung bursts, and the size of the episode changes as respiratory drive changes (for review see Kinkead, 1997). Lung burst frequency increases with SP and returns to baseline during antagonist D treatment (Davies *et al.*, 2008). Interestingly, both SP and antagonist D decrease the number of lung bursts per episode, while the episode frequency increases with SP and decreases during antagonist D application. This suggests that SP does not enhance respiratory drive in a way that increases the number of lung bursts per episode. SP-induced increases in drive manifest as more frequent episodes with fewer breaths; this in turn leads to an increase in lung burst frequency. A decrease in the non-ventilatory period caused by breaths occurring more frequently also contributes to the increase in lung burst frequency. NK1Rs are not only involved in respiratory-related output by increasing the number of lung bursts per minute, they also alter the ability to produce episodes with higher numbers of lung bursts. These data support the theory discussed by Kinkead *et al.* (1994) that pattern formation is an intrinsic property of the respiratory control network.

Baclofen eliminates clustering without changing respiratory drive; lung burst frequency remains the same on average and has been proposed to directly affect the mechanism responsible for clustering breaths (Straus *et al.*, 2000). In my study, although lung bursts per episode decreased, the overall lung burst frequency increased with SP. This suggests that SP does not act directly on the mechanisms responsible for pattern

formation, but rather influences respiratory drive and has a secondary influence on episodic pattern formation.

DAMGO decreases lung burst frequency (see Chapter 1) while also decreasing the number of lung bursts per episode and the episode frequency. While there is a change in pattern, as there are fewer bursts per episode, there is also an overall decrease in respiratory drive indicated by the decrease in lung burst and episode frequencies. This suggests that DAMGO, like SP, does not directly influence the mechanisms responsible for pattern formation, but influences the overall respiratory drive and has an indirect influence on episode formation. Together, these data support the theory proposed by Oka (1958a; 1958b) that the site responsible for pattern generation is different from that responsible for rhythm generation.

2.4.2 Changes in lung burst parameters

Changes in lung burst parameters including duration, amplitude and area indicate changes in respiratory drive caused by influences on the respiratory rhythm generator (RRG). SP significantly decreases lung burst duration. This effect is not surprising when evaluated with the significant increase in lung burst frequency. The decrease in lung burst duration allows for subsequent breaths to follow at a quicker rate, thus increasing frequency. In anurans, lung burst area has been associated with tidal volume (McAneney & Reid, 2007). The increase in area during SP treatment despite the decrease in lung burst duration is attributed to the increase in lung burst amplitude. Despite the shorter duration, the lung bursts are larger than at baseline, allowing for a greater area. The significant increase in area during antagonist D is attributed to the larger duration, which returned to baseline levels, together with the larger amplitude, which remained constant after SP application.

Duration and amplitude increases during DAMGO treatment, leading to an increase in area. These parameters may increase to compensate for the significant decrease in lung burst frequency. In order to maintain a normal level of inspired air during respiratory depression, the RRG compensates by increasing the duration and amplitude of each breath.

This study has shown that NK1Rs and μORs influence episodic ventilation by affecting the overall respiratory rhythm generation rather than directly affecting the mechanisms involved in pattern formation. These data support claims that pattern formation is an intrinsic property of the respiratory control network and may arise from an area independent of the RRG. Further research is needed to completely characterize the mechanisms behind this complex pattern.

2.5 Acknowledgements

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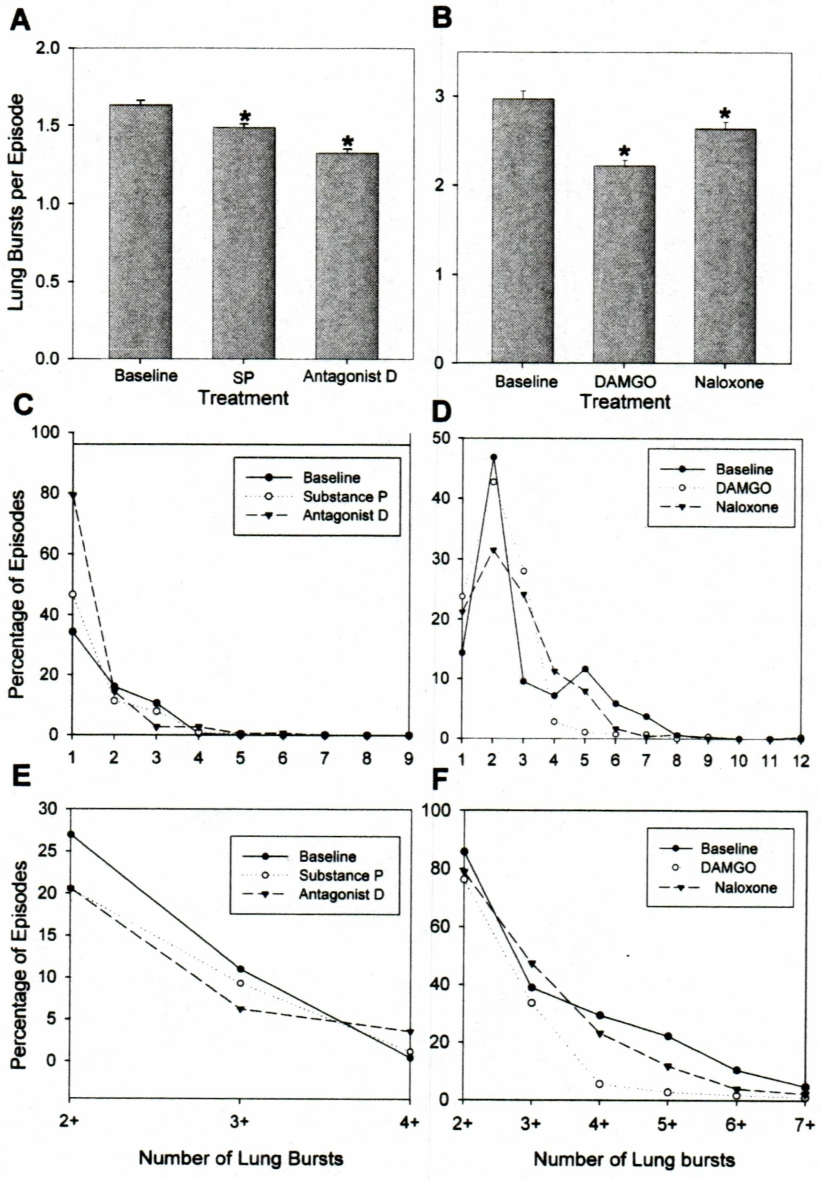

Figure 2-1. Effect of SP and DAMGO on the number of lung bursts per episode exhibited by *in vitro* brainstem preprations from juvenile bullfrogs. (A) Lung bursts per episode significantly decreased from baseline with bath application of SP. Bursts per episode exhibited a further significant decrease during antagonist D application. (B) Lung burst per episode significantly decreased from baseline with application of DAMGO. Bursts per episode increased during naloxone application. (C) Baseline, SP and antagonist D treatments all have the highest percentage of episodes containing single bursts. (D) Baseline, DAMGO and naloxone treatments all had the highest percentage of episodes containing two bursts. (E) The slope of the line for the percentage of episodes containing 2+ and 3+ bursts decreased faster for baseline and antagonist D than for substance P. (F) The slope of the line for the percentage of episodes containing 2+ bursts decreased faster for DAMGO treatment than for baseline and naloxone. All data are means ± S.E.M. for n = 5-6 animals. Asterisks indicate a significant difference from all other treatments ($P < 0.05$).

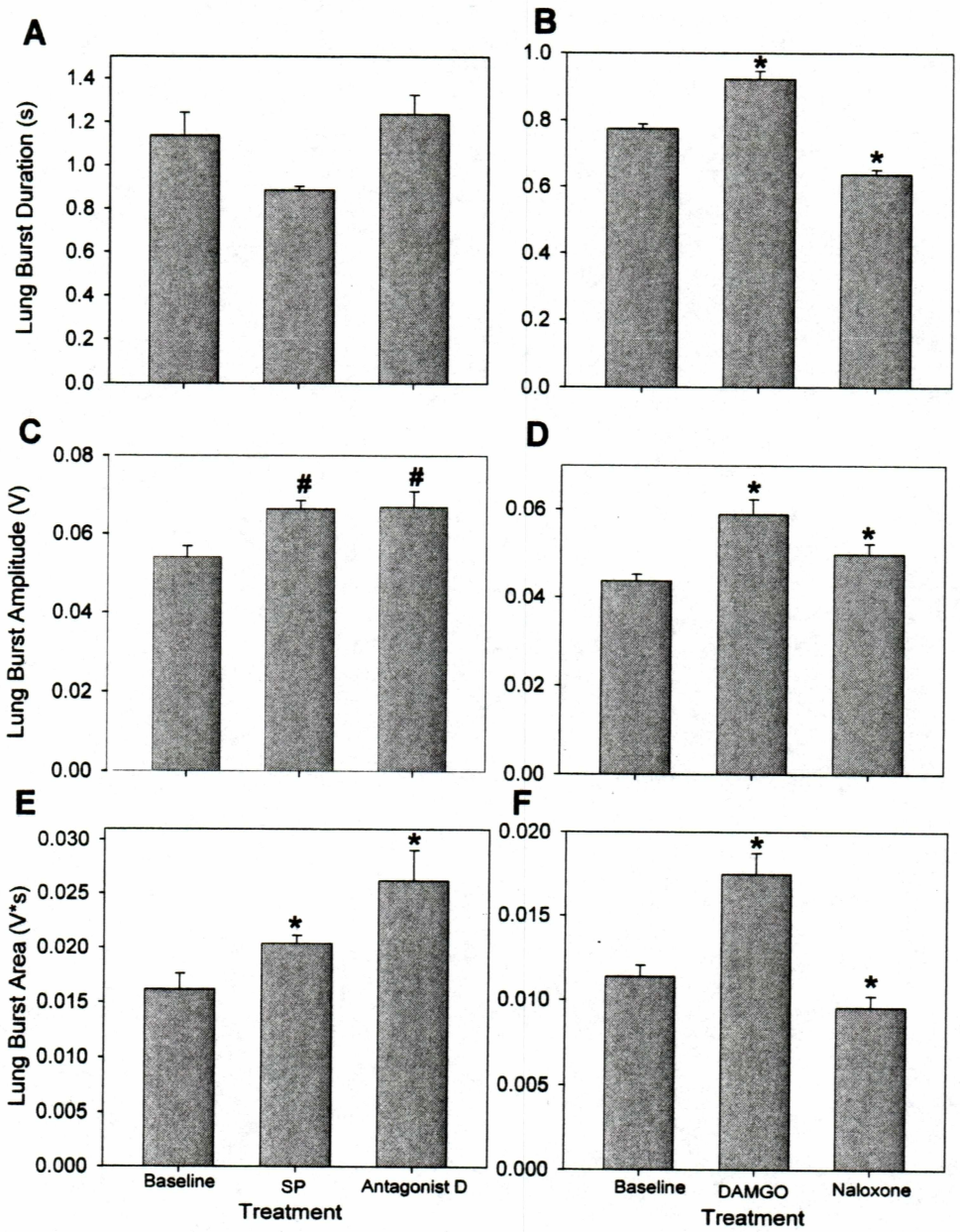

Figure 2-2. Effect of SP and DAMGO on lung burst parameters exhibited by *in vitro* brainstem preparations from juvenile bullfrogs. (A) SP decreased mean lung burst duration, which subsequently increased to baseline during antagonist D treatment. (B) Duration increased with DAMGO application and decreased to baseline during naloxone. (C) SP increased lung burst amplitude, which remained constant through antagonist D treatment. (D) DAMGO increased amplitude, which then decreased during naloxone application. (E) Lung burst amplitude increased with SP and antagonist D treatments. (F) Area of lung bursts increased throughout DAMGO exposure and decreased to near baseline levels during naloxone treatment. All data are means ± S.E.M. for n = 5-6 animals. Asterisks indicate a significant difference from all other treatments; number signs indicate a significant difference from baseline ($P < 0.05$).

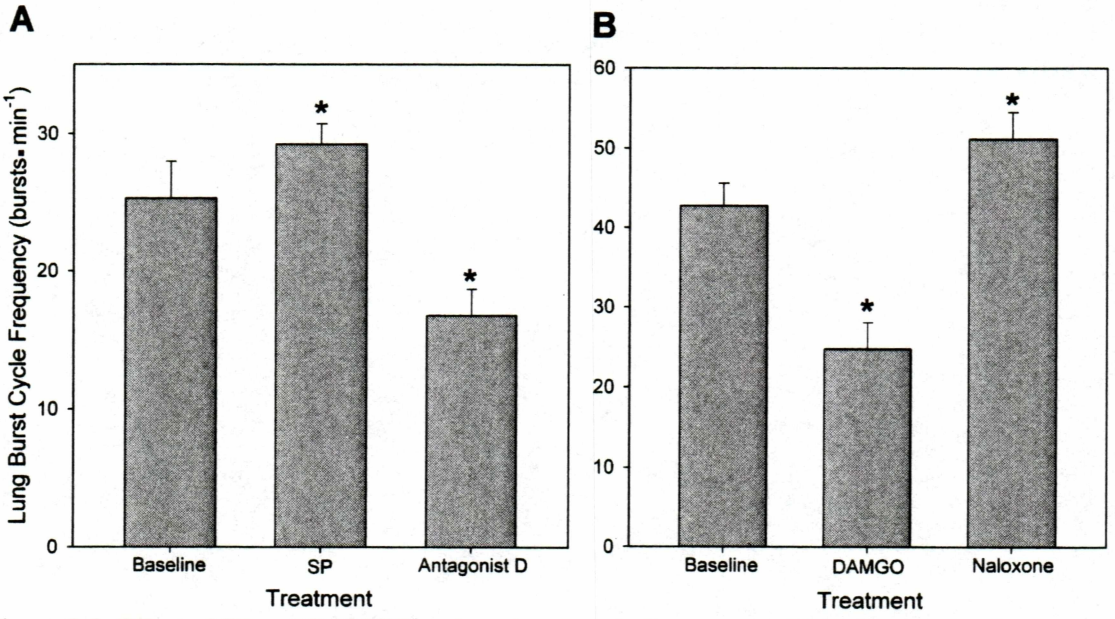

Figure 2-3. Effect of SP and DAMGO on lung burst cycle frequency exhibited by *in vitro* brainstem preparations from juvenile bullfrogs. (A) SP increased lung burst cycle frequency (BCF), which decreased below baseline levels during application of antagonist D. (B) BCF decreased during DAMGO treatment and increased above baseline levels during naloxone application. All data are means ± S.E.M. for n = 5-6 animals. Asterisks indicate a significant difference from all other treatments ($P < 0.05$).

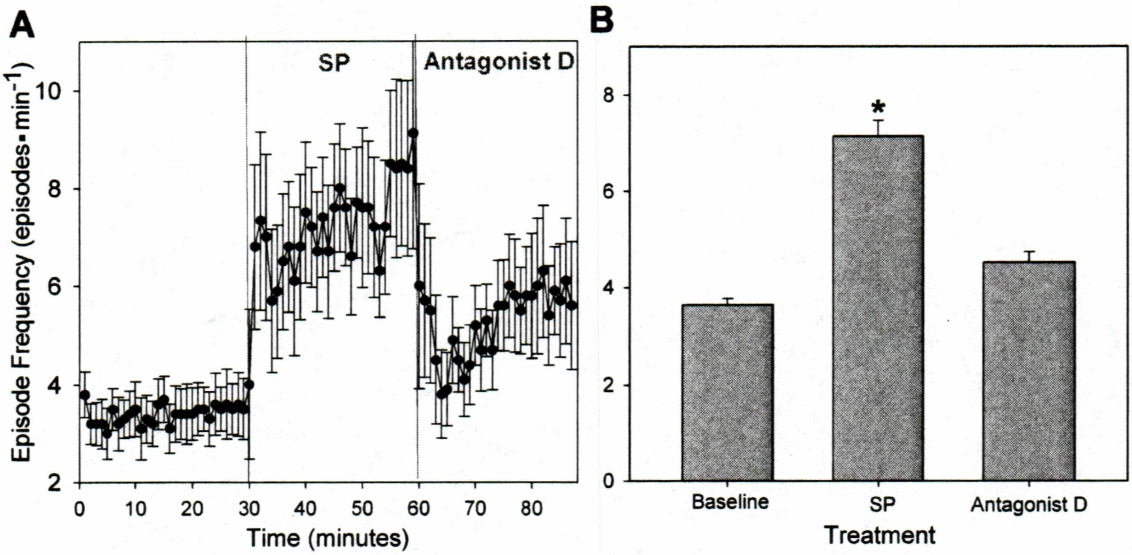

Figure 2-4. Effect of SP and antagonist D on episode frequency exhibited by *in vitro* brainstem preparations from juvenile bullfrogs. (A) Episode frequency for baseline, substance P and antagonist D treatment. (B) SP significantly increased episode frequency, which returned to baseline levels during antagonist D treatment. All data are means ± S.E.M. for n = 5-6 animals. Asterisk indicates a significant difference from all other treatments ($P < 0.05$).

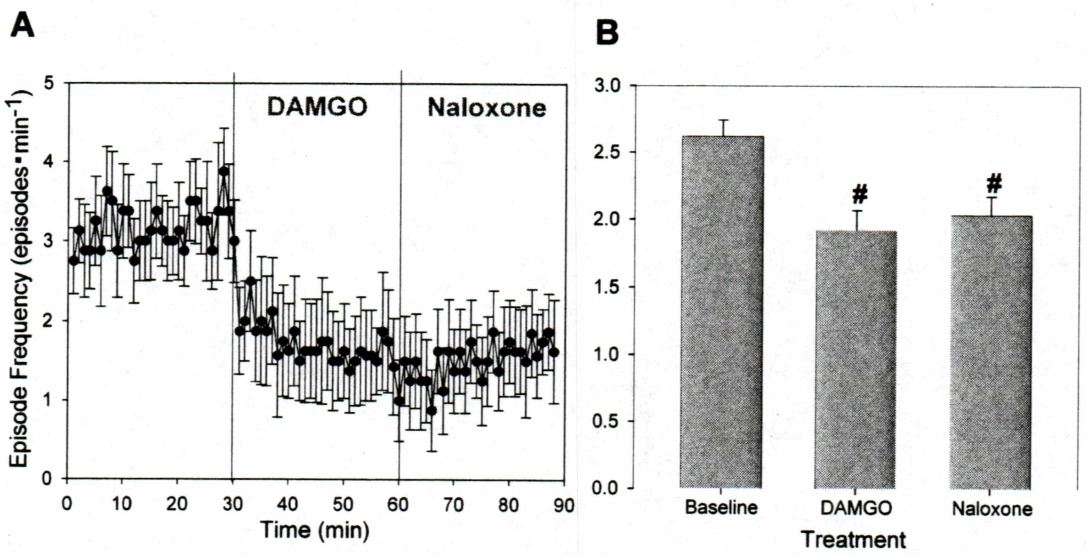

Figure 2-5. Effect of DAMGO and naloxone on episode frequency exhibited by *in vitro* brainstem preparations from juvenile bullfrogs. (A) Episode frequency for baseline, DAMGO and naloxone. (B) DAMGO significantly decreased episode frequency. All data are means ± S.E.M. for n = 5-6 animals. Number signs indicate a significant difference from baseline ($P < 0.05$).

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GENERAL CONCLUSIONS

This study verifies the similarity of the juvenile bullfrog and tadpole lung burst responses to substance P (SP), antagonist D, DAMGO and naloxone. Despite this physiological similarity, double-label immunofluorescence of neurokinin-1 (NK1R) and μ-opioid (μORs) receptors indicate a location of a respiratory rhythm generator (RRG) for juvenile bullfrogs but not tadpoles. The bullfrog location is closely associated with lung ventilation and is potentially homologous to the mammalian preBötzinger complex (PBC) RRG. I have also shown that NK1R and μOR neuromodulators influence the episodic ventilatory pattern in bullfrogs. SP, antagonist D, DAMGO and naloxone influence the number of lung bursts per episode, lung burst parameters, burst cycle frequency (BCF) and episode frequency.

Buccal and lung bursts are timed to one another in the isolated juvenile bullfrog brainstem preparation (Wilson *et al.*, 2002). One site, located between the auditory and glossopharyngeal nerves is essential for lung bursts but not buccal bursts, and it has been called the lung area (Wilson *et al.*, 2002). Another site, at the level of the vagus nerve, is essential for buccal bursts but not lung bursts (Wilson *et al.*, 2002), and it is called the buccal area.

Application of the μOR agonist DAMGO to juvenile bullfrog brainstem preparations *in vitro* results in a profound decrease in lung ventilation, suggesting that μORs have a role in modulating lung respiratory rhythm. These results are consistent with those of Vasilakos et al. (2005) who showed that the overall frequency of lung bursts in metamorphic and juvenile tadpole brainstems decreased with application of DAMGO. These authors found that buccal burst frequency remains constant in both stages of animals during DAMGO application. In intact frogs, morphine injection significantly decreases lung ventilation, but the total number of ventilatory events (buccal plus lung) remains constant (Vasilakos *et al.*, 2005); therefore, buccal ventilation replaces lung

ventilation. Data from my study confirm the juvenile response identified by Vasilakos and coworkers (2005) and characterize the same response in early-stage tadpoles.

Mammalian treatment with DAMGO produces results consistent with the anuran response. Following application of DAMGO, rat respiration gradually slows in a phenomenon known as quantal slowing, and returns to control values during application of naloxone (Mellen *et al.*, 2003). Those results led to the conclusion that PBC inspiratory neurons are driven in an opiate-sensitive manner by pre-inspiratory neurons during the expression of respiratory rhythm. Opiate-induced quantal slowing in anurans suggests a similar network. Homologies between the PBC and lung RRG have been proposed based on these findings (Vasilakos *et al.*, 2005).

SP modulates the activity of the RRG in newborn rats (Ptak *et al.*, 1999), and changes in the level of SP in the nucleus tractus solitarius have suggested a role for SP in regulating the onset of breathing (Srinivasan *et al.*, 1991). *In vitro* brainstem preparations of the newborn rat exhibit increased respiratory frequency with SP treatment (Yamamoto *et al.*, 1992). The significant increase in lung frequency with bath application of SP to juvenile frog and tadpole brainstem preparations suggests that the NK1Rs play a role in regulating respiratory rhythm throughout development.

The mammalian response to SP and DAMGO suggests that colocalization of NK1Rs and μORs will mark the PBC. High densities of NK1Rs within the PBC are used to locate the complex in brainstem slices (Gray *et al.*, 1999). The PBC NK1Rs colocalize with μORs (Gray *et al.*, 1999). Similar respiratory responses of anurans and mammals to SP and DAMGO suggest that colocaliztion of these receptors will mark the putative lung RRG of anurans. Lack of buccal response to opiates suggests that colocalization of these receptors will not mark the putative buccal RRG in brainstem slices.

One distinct site of colocalization was found caudal to the facial nerve, and is located near the bullfrog facial nucleus, an area purportedly involved in anuran respiratory rhythm generation. Lack of receptor colocalization in developing tadpole brainstems suggests that during development, there is a change in receptor expression leading to formation of the putative lung RRG. Throughout development, NK1R expression does not change. In contrast, the μOR expression increases throughout development. The lack of receptor colocalization during development may be attributed to the lack of μOR expression in tadpole stages. Interestingly, the early-stage tadpole response to SP and DAMGO is the same as that for juvenile bullfrogs. Because the neuromodulator exposure is through bath application, this indicates that early-stage tadpoles have NK1Rs and μORs, but does not indicate the receptors location. Nano-injection into specific areas in the brain would reveal specific receptor locations.

NK1R and μOR neuromodulators influence not only lung frequency but also characteristics of the episodic breathing pattern. Episodes can consist of any number of lung bursts, and the size of the episode changes with the level of respiratory drive. Though SP and antagonist D increase and decrease both lung burst frequency and BCF, respectively, they both decrease the number of lung bursts per episode. This suggests that SP does not enhance respiratory drive in a way that increases the number of lung bursts per episode; rather it increases the overall drive to take more frequent breaths. The increase in BCF is due to the decrease in the non-ventilatory period caused by more frequent breaths. NK1Rs are involved not only in respiratory-related output by increasing the number of lung bursts per minute, but also alter the ability to produce episodes with higher numbers of lung bursts. These data support the claim that pattern formation is an intrinsic property of the respiratory control network.

Baclofen eliminates clustering without changing respiratory drive, i.e., lung burst frequency remains the same. Baclofen, therefore, has been proposed to directly affect the

mechanisms responsible for episode formation (Straus *et al.*, 2000b). In my study, although the number of lung bursts per episode decreases, the overall lung burst frequency increases with SP, suggesting that SP does not act directly on the mechanisms responsible for pattern formation, but influences respiratory rhythm generation.

DAMGO decreases the lung burst frequency while decreasing the number of lung bursts per episode and the episode frequency. While there is a change in pattern, there is also an overall decrease in respiratory drive indicated by the decrease in lung burst and episode frequencies. This suggests that, like SP, DAMGO does not directly influence the mechanisms responsible for pattern formation, but influences the overall drive to produce respiratory rhythm. Together, these data support the hypothesis that the site responsible for pattern generation is different from that responsible for rhythm generation.

Changes in lung burst parameters including duration, amplitude and area indicate changes in respiratory drive caused by influences in the RRG. SP significantly decreases lung burst duration, which is not surprising when evaluated with the increase in lung burst frequency. The decrease in duration allows for subsequent breaths to follow at a faster rate, thus increasing frequency. Lung burst area has been associated with tidal volume in anurans (McAneney & Reid, 2007), and the increase in area during SP treatment despite the decrease in duration is attributed to the increase in amplitude. Despite the shorter duration, the lung bursts are stronger allowing for greater tidal volume.

Duration and amplitude of lung bursts increase during DAMGO exposure, leading to an increase in area. The parameters may have been increased to compensate for the significant decrease in frequency. In order to retain a normal level of inspired air during respiratory depression, the RRG compensates by increasing the duration and amplitude of each infrequent breath.

Overall, this study confirms that the juvenile bullfrog and tadpole *in vitro* brainstem preparations respond to SP and DAMGO in a manner similar to those documented in mammalian preparations. I have established, using double-label immunofluorescence techniques, a proposed site in the juvenile bullfrog brainstem of the putative lung RRG. Furthermore, I have shown that NK1Rs and μORs influence episodic breathing by affecting the overall respiratory rhythm generation rather than directly acting on the mechanisms involved in pattern formation. These data support claims that pattern formation is an intrinsic property of the respiratory control network and may arise from an area independent of the RRG.

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